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Attorney's Docket No. 178-289

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Assistant Commissioner for Patents
Box Patent Application
Washington, D.C. 20231



UTILITY PATENT APPLICATION TRANSMITTAL

Sir:

Transmitted herewith for filing is the patent application of:

First Named Applicant (or Applicants): Chu et al.

Title of Application:

**POLYMER SOLUTION FOR SEPARATION OF CHARGED
MACROMOLECULES BY ELECTROPHORESIS**

1. Type of Application (37 C.F.R. 1.53(b))

This application is a(n):

- ☒ Original (nonprovisional) application.
- ☐ Continuing application:
- ☐ Divisional ☐ Continuation ☐ Continuation-in-Part (CIP)
- of Serial No. 08/ _____, filed on _____.

CERTIFICATION UNDER 37 CFR 1.10

I hereby certify that this New Application Transmittal and the documents referred to as enclosed herein are being deposited with the United States Postal Service on this date, June 5, 2000, in an envelope as "Express Mail to Addressee" Mailing Label Number EL457629995US, addressed to the Commissioner of Patents and Trademarks, Washington, D.C. 20231.

Melinda A. Kerrigan
Name of person mailing paper


Signature of person mailing paper

2. Enclosed Papers Required to Obtain Application Filing Date under 37 CFR 1.53(b)

<u>34</u>	Pages of specification		
<u>5</u>	Pages of claims		
<u>1</u>	Pages of Abstract		
<u>12</u>	Sheets of drawings	<input type="checkbox"/> Formal	<input checked="" type="checkbox"/> Informal

3. Oath or Declaration

- ☒ Newly executed Oath or Declaration (original or copy) is enclosed.
- ☐ Copy of Oath or Declaration from prior application 0 / _____ (37 C.F.R. 1.63(d)).
- ☐ The entire disclosure of the prior application, from which a copy of the oath or Declaration is supplied, is considered as being a part of the disclosure of the accompanying application and is hereby incorporated by reference therein.
- ☐ With Power of Attorney ☐ Without Power of Attorney

4. Additional Papers Enclosed

- ☒ Return Receipt Postcard (specifically itemized) (M.P.E.P. § 503).
- ☐ Preliminary Amendment.
- ☐ Information Disclosure Statement (37 CFR 1.98).
- ☐ Form PTO-1449 ☐ Copies of IDS Citations
- ☐ Nucleotide and/or Amino Acid Sequence Listing computer-readable copy, paper copy, and statement verifying identity of computer-readable and paper copies.
- ☐ Certified Copy of Priority Document(s).
- ☐ Verified translation of non-English language application (37 C.F.R. 1.52(d)).
- ☐ Other: _____.

5. Assignment

- ☐ Newly Executed assignment with Recordation Cover Sheet (Form PTO-1595).
- ☐ Copy of Assignment from prior application No. 08 / _____.

6. Fee Calculation (37 CFR 1.16)

Regular Application (37 CFR 1.16(a))

Basic Fee \$690.00

FEES FOR CLAIMS AS FILED					
Number filed	Number extra		Rate		
Total Claims (37 CFR 1.16 (c))	27	- 20	= 7	x \$ 18.00	= \$ 126.00
Independent Claims (37 CFR 1.16(b))	2	- 3	= 0	x \$ 78.00	= \$ 0.00
Multiple Dependent Claims (37 CFR 1.16(d))			+ \$ 260.00	=	\$ 0.00
			Fee Calculation for Extra Claims		\$ 126.00

☐ Amendment canceling extra claims enclosed.

☐ Amendment deleting multiple-dependencies enclosed.

Total Filing Fee Calculation \$ 816.00

7. Small Entity Statement

☒ Verified Statement that this is a filing by a small entity under 37 CFR 1.9 and 1.27:

☐ is enclosed.

☐ will follow.

☐ Status as a small entity was claimed in prior application 08/_____, from which benefit is being claimed for this application under:

☐ 35 U.S.C. 119(e),

☐ 35 U.S.C. 120,

☐ 35 U.S.C. 121,

☐ 35 U.S.C. 365(c),

and which status as a small entity is still proper and desired.

☐ A copy of the verified statement in the prior application is enclosed.

Filing Fee Calculation (50% of Filing Fee calculated in Item 6 above)

\$ 408.00

8. Fee Payment

☐ Not enclosed. No filing fee is to be paid at this time.

☒ Enclosed:

<input checked="" type="checkbox"/>	Basic filing fee (Item 6 or 7 above)	\$ <u>408.00</u>
<input checked="" type="checkbox"/>	Fee for recording Assignment (\$40.00 (37 CFR 1.21(h)))	\$ <u>40.00</u>
<input type="checkbox"/>	Processing and retention fee (\$130.00 (37 CFR 1.53(d) and 1.21(l)))	\$ _____
Total fees enclosed		\$ <u>448.00.</u>

9. Method of Payment of Fees

☒ Check in the amount of \$ 448.00.

☐ Charge Deposit Account No. 08-2461 in the amount of \$ _____.
A duplicate of this transmittal is enclosed.

10. Authorization to Charge Additional Fees

☒ The Commissioner is hereby authorized to charge the following additional fees by this paper and during the entire pendency of this application to Deposit Account No. 08-2461:

- ☒ 37 CFR 1.16(a), (f), or (g) (filing fees)
- ☒ 37 CFR 1.16(b), (c), and (d) (presentation of extra claims)
- ☒ 37 CFR 1.16(e) (surcharge for filing the basic fee and/or declaration at a date later than the filing date of the application)
- ☒ 37 CFR 1.17 (application processing fees)

A duplicate of this transmittal is enclosed.

Applicant or Patentee: Chu et al.

Serial or Patent No.: Unassigned

Filed or Issued: Herewith

For: **POLYMER SOLUTION FOR SEPARATION OF CHARGED
MACROMOLECULES BY ELECTROPHORESIS**

**VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY
STATUS (37 CFR 1.9(f) and 1.27(d))--NONPROFIT ORGANIZATION**

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below:

NAME OF ORGANIZATION The Research Foundation at State University of New York
ADDRESS OF ORGANIZATION SUNY at Stony Brook, N5002 Melville Memorial Library
Stony Brook, New York 11794-3368

TYPE OF ORGANIZATION:

- ☐ UNIVERSITY OR OTHER INSTITUTION OF HIGHER EDUCATION
- ☒ TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE
(26 USC 501 (a) and 501 (c)(3))
- ☐ NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF
STATE OF THE UNITED STATES OF AMERICA
(NAME OF STATE) _____
(CITATION OF STATUTE) _____
- ☐ WOULD QUALIFY AS TAX EXEMPT UNDER INTERNAL REVENUE
SERVICE CODE (26 USC 501 (a) and 501 (c)(3)) IF LOCATED IN THE
UNITED STATES OF AMERICA
- ☐ WOULD QUALIFY AS NONPROFIT SCIENTIFIC OR EDUCATIONAL
UNDER STATUTE OF STATE OF THE UNITED STATES OF AMERICA IF
LOCATED IN THE UNITED STATES OF AMERICA
(NAME OF STATE) _____
(CITATION OF STATUTE) _____

I hereby declare that the nonprofit organization identified above qualifies as a nonprofit organization as defined in 37 CFR 1.9(e) for purposes of paying reduced fees under Section 41(a) and (b) of Title 35, United States Code with regard to the invention entitled

**POLYMER SOLUTION FOR SEPARATION OF CHARGED
MACROMOLECULES BY ELECTROPHORESIS**

by inventors Dr. Benjamin Chu, Dr. Liguog Song, Dr. Dufei Fang, Dehai Liang and
Dr. Tianbo Liu

described in

- ☒ the specification filed herewith
☐ application serial no. 08/_____, filed
☐ patent no. _____, issued

I hereby declare that rights under contract or law have been conveyed to and remain with the nonprofit organization with regard to the above identified invention.

If the rights held by the nonprofit organization are not exclusive, each individual, concern or organization having rights to the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who could not qualify as a small business concern under 37 CFR 1.9(d) or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

*NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27).

NAME _____

ADDRESS _____

☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☐ NON PROFIT ORGANIZATION

NAME _____

ADDRESS _____

☐ INDIVIDUAL ☐ SMALL BUSINESS CONCERN ☐ NON PROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b)).

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING

John C. Petersen

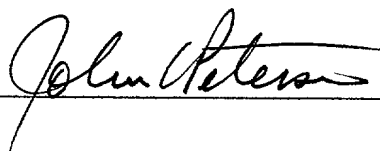
TITLE OF PERSON SIGNING

Director

ADDRESS OF PERSON SIGNING

The Research Foundation of State University of NY
SUNY at Stony Brook, N5002 Melville Memorial Library
Stony Brook, New York 11794-3369

SIGNATURE



DATE

5-25-00

**POLYMER SOLUTION FOR SEPARATION OF CHARGED
MACROMOLECULES BY ELECTROPHORESIS**

This invention was made with Government support under Grant No. 2R01HG0138604
5 awarded by the National Human Genome Research Institute. The Government has certain rights
in the invention.

BACKGROUND OF INVENTION

The present invention relates generally to the art of separating charged molecular
species, and, in particular, to separation media that are used for capillary electrophoresis.

10 Gel electrophoresis is one of the most widely used separation techniques in the
biologically related sciences. Charged molecular species such as proteins, peptides, nucleic
acids and oligonucleotides are separated by causing the species to migrate in a buffer medium
under the influence of an applied electric field. The buffer medium normally is used in
conjunction with a low to moderate concentration of an appropriate gelling agent, such as for
15 example, agarose or cross-linked polyacrylamide, to promote the separation and to minimize
the occurrence of mixing of the species being separated.

Until recently, electrophoretic separations were conducted in gel slabs or open gel beds
that were typically fabricated of agarose or cross-linked polyacrylamide material. More

recently, capillary electrophoresis ("CE") using a polymer gel or solution as a separation medium has been used for the separation of DNA. Capillary electrophoresis techniques combined with photometric detection methods have allowed the automation and rapid quantitative analysis of charged molecules. Furthermore, capillary electrophoresis can provide
5 quantitative information about a sample using very small amounts of the sample, gel (or polymer solution) and buffer relative to traditional slab gel processes. Moreover, high-resolution separation of charged macromolecules having different effective charges have been achieved.

Typically, the capillary columns used in capillary gel electrophoresis are fabricated from
10 fused silica tubing having diameters on the order of 25 μm to 200 μm and lengths from about 30 cm to about 200 cm. The column interior is filled with buffer and gel separation medium and electrophoretic techniques are used to separate charged molecular species.

Although the pore size of cross-linked polymer gels used for capillary electrophoresis can be controlled by the amount of monomers and cross-linked reagents, polymer gels have been
15 found to be inconvenient as separation media for large scale DNA sequencing analysis due to the instability, irreproducibility and difficulty in controlling the polymerization process inside the capillary tubing.

The inability of many separation media to bind directly to the inner wall of the capillary tubes is a major problem for capillary electrophoresis methods because it creates an electro-
20 osmotic flow when an electric field is applied during electrophoresis. Such migration results

in an unsatisfactory separation of the constituent parts of the sample. Traditional methods aimed at preventing electro-osmosis include introducing a compound that binds to the inner surface of a capillary tube wall and that is compatible with the separation medium prior to injecting the separation medium into the tube. For example, U.S. Patent No. 5,447,617 to Shieh
5 describes covalently bonding polybutadiene to the inner surface of a capillary tube, introducing acrylamide monomers therein and co-polymerizing the acrylamide with the polybutadiene. Such precoating techniques, however, are time consuming, inconvenient and costly.

Another problem encountered in conventional capillary gel electrophoresis results from the use of polyacrylamide-based separation media. Such media are injected into the capillary
10 tube in unpolymerized form. Polymerization of the polyacrylamide is then induced within the capillary tube by well known methods, such as ultraviolet radiation and chemical catalysts. Such methods are characterized by a lack of uniformity in the pore size distribution of the polymer network formed, and by incomplete polymerization.

The irreversible nature of the polymerized polyacrylamide gel also causes numerous
15 problems when a polyacrylamide-based separation medium is used in capillary gel electrophoresis methods. Once the polyacrylamide is polymerized within a capillary electrophoresis tube, the polymerized gel cannot be easily removed from the capillary tube after electrophoresis.

Capillary electrophoresis ("CE") provides numerous advantages over conventional slab
20 gel electrophoresis for DNA separation. The use of fused silica capillaries with inner diameters

of less than 100 μm enables CE to operate at very high separation voltages and offers fast separation, high efficiency and increased resolution. In addition, rigid gels, which are normally used in slab gel electrophoresis because of their anti-convection ability, are not needed in capillary electrophoresis. Cross-linked polyacrylamide ("PAM") gels, which are widely used in conventional slab gel electrophoresis, were initially used in capillary electrophoresis. Despite successful results, i.e., 700 bases read lengths with resolution of 0.5 for DNA sequencing in about 230 minutes, PAM gels have encountered problems due to bubble formation, gel inhomogeneity, and short lifetime of the capillary.

Accordingly, attempts have been made to use nonpolymerized separation media for capillary electrophoresis. For example, U.S. Patent No. 5,468,365 to Menchen et al. describes an electrophoresis medium having a matrix of aggregated copolymers dispersed in an aqueous medium. The polymer matrix of the '365 patent is described as a dispersion of one substance (micelles) in another substance (water). In such a dispersion, the particles are formed by the association or aggregation of molecules having both hydrophilic and hydrophobic regions. The copolymers of the '365 patent form a polymer matrix having a relatively uniform mesh size which is believed to be related to the regular, i.e., substantially uniform spacing between adjacent hydrophobic polymer segments.

A number of different polymers have been used in CE methods to separate DNA fragments. Many of these polymers are modified polysaccharides, such as, agarose, methylcellulose ("MC"), hydroxypropyl-methyl-cellulose ("HPMC"), hydroxyethylcellulose ("HEC"), hydroxypropylcellulose ("HPC"), glucomannan, galactonmannan, and dextran. Some

of them are synthesized polymers, such as, polyethylene glycol (“PEG”), polyethylene oxide (“PEO”), polyvinylpyrrolidone (“PVP”), polyvinylalcohol (“PVA”), polyacrylamide (“PAM”), poly-N-acryloyl-amino-ethoxyethanol (“PAAEE”), polyacryloylaminopropanol (“PAAP”), poly-N,N-dimethylacrylamide (“PDMA”), polyacrylamide-co-allyl-β-D-glucopyranoside (“P(AM/AG)”), and poly-N-(acryloylaminoethoxyethyl-β-D-glucopyranoside (“PAEG”).

Recently, polymers with viscosity dependent behavior have also been employed. One type of polymer was characterized by the collapse of molecules at high temperature, such as a copolymer of N,N-dimethylacrylamide and N,N-diethylacrylamide (“P(DMA/DEA)”), a copolymer of poly(N-isopropylacrylamide) (“PNIPAM”) densely grafted with short poly(ethylene oxide) (“PEO”) chains (“PNIPAM-g-PEO”), etc. Other polymers involved the formation of micelles, such as fluorocarbon end-capped polyethylene glycols, E₉₉P₆₉E₉₉ (with E being polyoxyethylene and P being polyoxypropylene), and n-dodecane-poly(ethylene oxide)-n-dodecane, etc. Each of these polymers has distinct advantages, but each also has inherent problems. For example, only several of them, such as HEC, PEO, PVP, PAM, PDMA, P(DMA/DEA), and fluorocarbon end-capped polyethylene glycols, have ever been used for DNA sequencing; and only HEC and high molecular weight PAM, PEO and PDMA have ever achieved a read length of greater than 500 bases.

Entangled polymer solutions, such as liquefied agarose, poly(acrylamide) (“PAM”), different kinds of cellulose, poly(ethylene oxide) (“PEO”) or poly(dimethylacrylamide), have been widely used as a DNA separation medium in CE with some success. High molecular weight (M_w) PAM has achieved 1,000-base read length in one run for single-stranded DNA in

run times of less than one hour. However, the PAM solution has two disadvantages: the injection is very difficult due to the very high viscosity, and the capillary inner wall has to be coated.

One base pair (“bp”) resolution of double-stranded DNA has been achieved by using PEO mixtures without coating the capillary inner wall, but the solution viscosity is too high for easy injection. In addition, the capillary channels have to be pretreated with a low viscosity PEO solution before the high viscosity matrix is injected. PEO mixtures have also achieved 1,000 base read length with run times of about seven hours. Other polymers that have been used for single-stranded DNA are poly(dimethylacrylamide) which has achieved about a 600-base read length with run times of about two hours and HEC which has achieved up to about 500 bases with run times of about one hour. A number of other polymers have been used which have achieved read lengths of less than 500 bases.

Mixtures of the same polymer, such as PEO, HEC and PAM, with different molecular weights and mixtures of two modified polysaccharides, i.e., agarose and HEC, have been found to produce a better resolution for both small and large DNA fragments. However, a mixture of two polymers with totally different chemical structures has never been successfully used. Kim et al. (Kim, Y., Yeung, E.S., *J.Chromatogr. A.*, 1997, 781, 315-325) tried to use a mixture of PEO and HPC for DNA sequencing and found the separation to be very poor. The failure was attributed to the incompatibility of the two polymers

The selection of the polymer used as the medium for DNA separation by capillary

electrophoresis is very important because the polymer determines the migration behavior and the resolution of DNA fragments. An equally important issue in DNA separation by capillary electrophoresis is the coating of the inner wall of a fused silica capillary, which suppresses both electro-osmosis and the adsorption of DNA fragments onto the capillary inner wall. A coating protocol using PAM covalently attached to the capillary through a Si-O-Si bond has been widely used. Several modifications of this protocol using PVA, PAAEE, PAAP or PDMA have also been used. Because of the hydrolysis tendency of the Si-O-Si bond in an alkaline environment, PAM, PAAEE, PAAP or PDMA have even been covalently attached to the capillary by a Si-C bond. Some commercially available GC capillaries from J & W Scientific Inc. with coatings such as DB-1 (100% dimethylpolysiloxane), DB-17 (50% diphenyl 50% dimethylpolysiloxane) and DB-Wax (100% polyethyleneglycols) have also been used. Despite the wide use of covalently coated capillaries, the coating methods often increase their cost by requiring *in situ* synthesis and give rise to problems such as capillary fouling, coating inhomogeneity, and limited lifetime.

Speed is also important in DNA separation. In the past, pBR322/Hae III has been separated with PAM, PAAEE and PAAP in about one hour. By modifying PAM to have less viscosity, shorter separation times of about tens of minutes have been achieved. With cellulose derivatives and PEO, the separation time is about twenty to thirty minutes. A 13-min separation of pBR322/Hae III has been achieved using the present invention with a separation medium of PNIPAM-g-PEO. Muller et al. has achieved an ultra-fast separation of pBR322/Hae III in 30 seconds with 1% MC. To achieve such a separation, a shorter effective separation length of 3

cm, a higher applied electric field strength of 800 V/cm and a narrower plug electrokinetic injection of 100 ms at 600 V/cm with a fast ramp power supply were employed. However, the separation of 434, 458 and 504 bp was hardly visible. And the partly separated 123/124 bp was a result of the structurally dependent migration at high electric field strengths rather than a result of single base pair resolution.

PAM gels have become less popular due to bubble formation, gel inhomogeneity, and short lifetime of the capillary. These problems have been eliminated in the present invention through the use of non-crosslinked polymer solutions. These polymer solutions can be replaced after each electrophoresis run if necessary, which makes CE particularly well suited for automation.

Accordingly, it would be desirable to provide a new separation medium for capillary electrophoresis methods, which has alleviated the bubble formation problem and reduced the inhomogeneity of the separation medium. It would also be desirable to provide a separation medium that has increased capillary lifetime and does not require coating of the interior surface of a capillary tube. In particular, it would be desirable to provide a separation medium that provides high resolution and is easy to apply and remove from various apparatus.

SUMMARY OF THE INVENTION

The present invention is a polymer solution for the efficient separation of charged macromolecules by electrophoresis that includes a plurality of polymers. These polymers are

different, do not phase separate when dissolved in solution and are entangled to form an interpenetrating network. In some embodiments, these polymers are neutral and water-soluble. Preferred polymer solutions of the present invention provide at least a 500-base read length in one run for a single-stranded DNA separation.

5 At least one of the polymers in the polymer solution is PAM, N-substituted PAM, N,N-disubstituted PAM, modified polysaccharides, PEO, PVP, PVA, PEG, or a random, a graft or a block copolymer based on the backbone monomer segments thereof. The nitrogen substitutes are C₁ to C₃ alkyl, hydroxyl-substituted C₁ to C₃ alkyl or methoxy-substituted C₁ to C₃ alkyl. The random, graft or block copolymer can be EPE-type Pluronics, P(DMA/DEA), PNIPAM-g-
10 PEO or P(AM/AG). The polysaccharides can be liquified agrose, MC, HEC, HPMC, HPC, glucomannan, galactonmannan and dextran.

 In a preferred embodiment, at least one of the polymers in the polymer solution is a silica-absorbing polymer that suppresses electrophoretic flow and charged
15 macromolecule-silica interactions. The silica-absorbing polymer can be PVP, PEO, EPE-type Pluronics, N-substituted PAM or N,N-disubstituted PAM. The nitrogen substitutes can be C₁ to C₃ alkyl, hydroxyl-substituted C₁ to C₃ alkyl, or methoxy-substituted C₁ to C₃ alkyl.

 The interpenetrating network polymer solution has a more expanded structural formation than the entanglement structure of a corresponding homopolymer solution and it has a larger effective size than that of a corresponding homopolymer solution. This represents an effective
20 entanglement network greater than that of the corresponding homopolymers. The

interpenetrating network can be prepared by synthesizing a first polymer in a matrix of a second polymer solution or by dissolving together the two polymers in a solvent.

Another embodiment of the present invention is a polymer solution for the efficient separation of charged molecules by electrophoresis that can provide at least a 500-base read length in one run for a single-stranded DNA separation. The polymer solution includes a plurality of stretched polymer chains that have polymer chain entanglement times greater than the corresponding linear homopolymer solution. The polymer chains include the same polymer or a plurality of different polymers. These polymer chains can entangle to form an interpenetrating network in solution. Preferred polymers are PAM and PVP or PDAM and PVP.

The polymer chains can include a random copolymer made up of a monomer taken from PAM, N-substituted PAM, N,N-disubstituted PAM, modified polysaccharides, PEO, PVP, PVA or PEG. The random copolymer can also include a silica-absorbing segment taken from PVP, PEO, EPE-type Pluronics, N-substituted PAM or N,N-disubstituted PAM, wherein nitrogen substitutes can be C₁ to C₃ alkyl, hydroxyl-substituted C₁ to C₃ alkyl, or methoxy-substituted C₁ to C₃ alkyl. In a preferred embodiment, the random copolymer is made up of AM and DMA.

The polymer chains can include a graft copolymer that includes a monomer taken from PAM, N-substituted PAM, N,N-disubstituted PAM, modified polysaccharides, PEO, PVP, PVA or PEG. The graft copolymer can also include a silica-absorbing segment taken from PVP, PEO, EPE-type Pluronics, N-substituted PAM or N,N-disubstituted PAM, wherein nitrogen substitutes can be C₁ to C₃ alkyl, hydroxyl-substituted C₁ to C₃ alkyl, or methoxy-substituted C₁ to C₃ alkyl. In a preferred embodiment, the graft copolymer includes PNIPAM-g-PEO.

Figures 2A-2D show the test results for a separation of pBR 322/Hae III DNA by CE in interpenetrating networks of a fixed amount of PVP and varying amounts of PAM.

Figure 3 shows the dependence of migration time of the 587 bp fragment for a separation of pBR 322/Hae III DNA by CE in interpenetrating networks of PAM and PVP.

5 Figures 4A-4D show the test results for a separation of pBR 322/Hae III DNA by CE in interpenetrating networks of PAM and PVP using different electric field strengths.

Figure 5 shows the separation times of pBR 322/MSP I using a separation medium of 2% PVP + 2% PAM.

10 Figures 6A and 6B are graphs of migration time versus length of DNA fragments for multiple runs of the same separation medium.

Figures 7A-&D show a comparison of the separation times for single-stranded DNA using P(AM/DMA) 2:1 and PAM.

Figures 8A and 8B show the separation time under optimum conditions for single-stranded DNA using P(AM/DMA) 3:1.

15 The percentages shown in the drawings are weight/volume percentages (w/v %).

DETAILED DESCRIPTION OF THE INVENTION

The present invention is a method of preparing a capillary electrophoresis separation medium that includes one or more polymers. For example, the separation medium is made up of two polymers, wherein the first and the second polymers are different. The separation medium can exist as interpenetrating networks, random copolymers and/or graft copolymers. One method of forming the interpenetrating network is by synthesizing the first polymer in the presence of the second polymer. The forced entanglement of two or more polymers can also form the interpenetrating network.

The separation medium that is made up of two (or more) polymers can be chosen from a broad range of water-soluble neutral polymers, such as agrose, MC, HPMC, HEC, HPC, PEG, PEO, PVP, PVA, PAM, PAAEE, PAAP, PDMA, P(AM/AG), PAEG, P(DMA/DEA), PNIPAM, PNIPAM-g-PEO, the Pluronics and fluorocarbon end-capped polyethylene glycols, etc. At least one of the two (or more) polymers should have dynamic surface coating ability.

In one embodiment, the first polymer is a (modified) polyacrylamide, preferably poly(acrylamide) (PAM). In other embodiments, the first polymer is a modified polysaccharide, such as hydroxyethylcellulose (HEC), a copolymer N,N-dimethylacrylamide and N,N-diethylacrylamide P(DMA/DEA) or a fluorocarbon end-capped polyethylene glycol.

The second polymer is preferably poly(vinylpyrrolidone), poly-N,N-dimethylacrylamide (PDMA) or polyethylene oxide (PEO). Other polymers which are reactive with the SiOH group of a fused silica compound can also be used. In a preferred embodiment, the first polymer is

poly(acrylamide) and the second polymer is poly(vinylpyrrolidone) or poly-N,N-dimethylacrylamide (PDMA). The separation methods of the present invention preferably achieve at least a 500-base read length in one run for single-stranded DNA fragments.

In addition, the present invention includes a method of preparing a capillary
5 electrophoresis separation medium having a selected mesh size. The method includes selecting a desired mesh size for the separation medium and then determining the ratio of the amount of a first polymer to the amount of a second polymer that is required to provide this mesh size, wherein the first and the second polymers are different and do not exhibit phase separation when dissolved together in a solvent. One method is to synthesize the first polymer in a solution of
10 the second polymer to form a separation medium that has interpenetrating networks of the first and second polymers.

The first polymer and the second polymer are the same as the polymers discussed above and the separation medium formed from these polymers achieves a read length of at least 500 bases in one run for single-stranded DNA fragments. For example, the weight ratio of the first
15 polymer (PAM) to the second polymer (PVP) is from about 1:12 to about 16:1, preferably from about 1:2 to about 2:1 and most preferably about 1:2.

The present invention is also a capillary electrophoresis separation system made up of entangled polymer chains that provide a mesh size effective to achieve a single-base resolution and at least 500 bases in one run for single-stranded DNA fragments. The polymer
20 entanglement in the interpenetrating network includes a first polymer and a second polymer

which are structured so that the polymer entanglement has a lower weight to volume ratio than the combined weight to volume ratios of the first polymer and the second polymer. In one embodiment, the first polymer is a (modified) polyacrylamide and the second polymer is reactive with the SiOH group of a fused silica compound.

5 In a preferred embodiment, the polymer entanglement used in the capillary electrophoresis separation system is an interpenetrating network of two or more polymers. Each polymer can be a linear homopolymer, a graft copolymer, a random copolymer, a weakly cross-linked microgel or a combination of two or more thereof. The polymer entanglement can include an interpenetrating network that contains a synthesis of the first polymer and second
10 polymer, wherein the first and said second polymers are different and do not phase separate when dissolved together in a solvent. The graft copolymer is made up of short chain polymers having a first monomer grafted to a backbone polymer chain that contains a second monomer, wherein the first and second monomers are different. The random copolymer is a polymer chain having both a first monomer and a second monomer, wherein segments (or regions) of the
15 monomers tend to repel each other.

The separation media of the present invention are formed by combining two or more different neutral, hydrophilic, stable materials with at least one having surface active properties in an interpenetrating network in order to take advantage of the properties of each of the polymers. These interpenetrating networks have an appropriate mesh size and sufficient surface
20 activity to suppress electro-osmosis and DNA adsorption on the substrate containing the separation medium. Highly entangled interpenetrating networks require a minimal amount of

polymers in solution while achieving a large dynamic range for separation of macromolecular fragments. Consequently, charged macromolecular separation can occur in a relatively short run time. In order to attain structure for rapid separation, the combined polymer chains are more extended or "stretched" when compared to corresponding homopolymer chains in the same buffer solution. Thus, the separation medium of the present invention requires a smaller amount of polymer to produce a network of the same effective mesh size. The present invention also increases the polymer chain entanglement times in order to simulate a chemically cross-linked gel. This allows the separation medium of the present invention to separate larger size fragments.

The present invention uses the concept of interpenetrating networks, to combine two different polymer chains in such a manner that the chains are stretched beyond their normal conformation. Different-types of polymer chains tend to resist combining with each other. This is caused by the incompatibility of different polymer chains. The incompatibility forces the different polymer chains to move away from each other. It has been found that the exertion of these forces results in the polymer chains being extended or "stretched." The "stretched" polymer chain conformations have the same molecular weight as they did prior to being mixed to form the interpenetrating network. It has been found that these "stretched" polymer chains provide a more effective separation medium.

Extended polymer chains and increased polymer chain entanglement times can also be accomplished by grafting a small amount of short-chain polymer having a different kind of monomer to the backbone of a main chain polymer. This procedure is described in two articles

5 titled: “Copolymers of Poly(*N*-isopropylacrylamide) Densely Grafted with Poly(ethylene oxide) as High-Performance Separation Matrix of DNA,” Liang, D., Zhou, S., Song, L., Zaitsev, V., and Chu, B., *Macromolecules*, Vol. 32, No. 19, pp. 6326-6332 (1999) and “Poly(*N*-isopropylacrylamide) - *g*-poly(ethyleneoxide) for high resolution and high speed separation of DNA by capillary electrophoresis,” Liang, D., Song, L., Zhou, S., Zaitsev, V., and Chu, B., *Electrophoresis*, Vol. 20, pp. 2856-2863 (1999). Both of these references are incorporated herein in their entirety.

10 Grafting short chains of a different kind to the polymer backbone is a tedious procedure because each change in the length of the side chain and the density of the side chain requires a complete synthetic step and characterization. However, polymer chains produced by this grafting method have increased polymer chain entanglement times, which provide a better separation medium for capillary electrophoresis.

15 In another embodiment, the separation medium can be extended by using random copolymers that include an appropriate ratio of two different monomers in the same polymer backbone chain. The various short “blocks” of different monomer segments in the backbone tend to repel each other, which results in the overall random copolymer chains being more extended in some regions. In addition, the different polymer segments increase the intrachain entanglement points in other regions of the polymer chain backbone. The local aggregation behavior causes the polymer chains to stay together for longer periods of time, further enhancing
20 the effectiveness of the separation medium.

The effectiveness of the separation medium can also be improved by selecting one monomer segment, which is more surface active than the other monomer segment due to a difference in hydrophobicity. This provides a more effective separation medium than a backbone polymer having monomer segments with similar hydrophobicity. An example of such a separation medium is a random copolymer of acrylamide and dimethyl acrylamide, which has shown a significant improvement in electrophoresis data when tested.

Chemical cross-linking is one method of increasing the entanglement times of polymer chains and such cross-linked polymers are particularly well suited for the separation of large size DNA fragments. Unfortunately, chemically cross-linked gels are not easy to handle and create several problems when used in capillary electrophoresis. The present invention takes advantage of the benefits that cross-linking provides by using a very weakly cross-linked microgel. The weakly cross-linked microgels of the present invention have some permanent cross-linking points and increase the effective polymer molecular weight of the separation medium, while avoiding the problems associated with polymers that have been extensively cross-linked. These very weakly cross-linked microgels improve the effectiveness of the separation media of the present invention. The very weakly cross-linked microgels can make up either one or both of the polymers used in forming the interpenetrating network of the separation medium.

The separation media of the present invention can use interpenetrating networks, graft copolymers, random copolymers, weakly cross-linked microgels or a combination of two or more of these methods to provide increased polymer entanglements without an increase in molecular weight. The increase in entanglement times provides better separation and the lower

polymer concentration of the medium allows faster run times. These characteristics are particularly useful in the field of capillary electrophoresis. Using the separation medium of the present invention, a read length of over 1000 bases in a sequencing buffer has been achieved in a short run time. The present invention combines the advantages of two dissimilar polymers, such as PAM and PVP, to provide an improved separation medium.

Two important issues in DNA separation by capillary electrophoresis are the selection of the separation medium and coating the inner wall of a fused silica capillary. The selection of the separation medium determines the migration behavior and the resolution of DNA fragments. The coating of the inner capillary wall insures the accuracy of the separation measurement by suppressing both electro-osmosis and the adsorption of DNA fragments. The present invention solves the problem of coating the inner capillary wall by using an alternative dynamic coating protocol. The dynamic coating protocol uses the molecular interaction (mainly hydrogen bond) between the Si-OH group of the capillary inner wall and the separation medium. Polymers, such as PEO, E₉₉P₆₉E₉₉, PDMA and PVP, have been found to provide the strongest interactions.

As a separation medium, PAM and PVP each have distinct advantages and drawbacks. Of all the polymers currently in use, PAM gives one of the highest resolutions for both double stranded and single stranded DNA fragments. The major problem encountered when using PAM as a separation medium in capillary electrophoresis is that PAM cannot dynamically coat the capillary inner wall. When compared with PAM, PVP produces significantly poorer results when used as a separation medium. However, PVP is much less viscous (a 4.5% weight/volume

(w/v) PVP with a molecular weight of 1 MDa has a viscosity of 27 cP at 30 °C) than PAM and dynamically coats the interior walls of the capillary when used as a separation medium for capillary electrophoresis.

The interpenetrating networks of the present invention are formed from two or more different polymers, which do not phase separate when they are dissolved together in a solvent. Instead, the essentially immiscible polymer chains have to be effectively entangled to form an interpenetrating network in solution. For example, PVP and PAM were formed into a network by synthesizing PAM in a PVP solution matrix. The present invention combines different polymers to take advantage of the desirable characteristics of each of the polymers. For example, PAM (a good separation medium) is combined with PVP (a separation medium which does not require precoating of the inner capillary wall) to provide an interpenetrating network of PVP and PAM which can dynamically coat the inner wall of a fused silica capillary and becomes a better separation medium than PAM.

The present invention uses a mixture of two polymers with different chemical structures to form interpenetrating networks that provide an improved separation medium and dynamically coat the inner capillary walls. The separation media take advantage of the characteristics of the different polymer structures to provide increased resolution and faster run times. Laser light scattering studies showed the formation of interpenetrating networks. The presence of interpenetrating networks is responsible for the better separation.

PAM gels have encountered problems due to bubble formation, gel inhomogeneity, and

short lifetime of the capillary. These problems have been eliminated in the present invention through the use of non-crosslinked polymer solutions. The interpenetrating networks of the present invention can be easily removed from the capillary because the polymers are not chemically bound together by cross-links. Thus, these polymer solutions can be replaced after each electrophoresis run if necessary, which makes the separation media of the present invention well suited for automated capillary electrophoresis.

For a given polymer, the optimization of separation conditions for capillary electrophoresis in DNA sequencing analysis depends on three primary factors: the polymer concentration, the sample injection amount and the electric field strength. The concentration of the polymer in the separation medium affects the resolution, the run time and the read length. Higher concentrations provide higher resolution, while lower concentrations provide lower viscosity, faster separation and longer read length. The optimum concentration would have sufficient resolution at the lowest possible concentration. The amount of sample injected affects the resolution as well as the signal to noise ratio. For optimum results, the injection amount should be kept to a minimum in order to provide high resolution and a sufficiently high signal to noise ratio. The electrical field strength is moderately high, about 150 V/cm. A higher electric field strength provides faster separation and higher resolution for smaller fragments. However, if the electric field strength is too high, larger fragments will have lower resolution and the read length will be shorter.

EXAMPLES

Capillary Electrophoresis Procedures

For the tests described in the examples, a 12-cm long fused silica capillary (Polymicro Technologies, Phoenix, AZ) with ID/OD of 100 or 50/365 μm was flushed with 1 M HCl for about 10 minutes. A detection window was opened at 2 cm from the cathodic end of the capillary by stripping off the polyimide coating with a razor blade. A gas tight syringe was then used to fill the capillary with the separation medium. Before each electrophoresis run, the capillary column was conditioned under an electric field strength of 200 V/cm until the current became stable (generally about 10 min). During this period, ethidium bromide migrated into the capillary from the buffer. The DNA sample was then electrokinetically injected into the capillary at an electric field strength of 50 V/cm for 3 seconds. The running electric field strength was generally 100 V/cm, although some tests were carried out at different field strengths.

Static light scattering (SLS) and dynamic light scattering (DLS) measurements were used to characterize the polymer systems in aqueous solution. The measurements were made using a standard laboratory-built light scattering spectrometer, which was capable of both SLS and DLS measurements over an angular range of 15-140°. The spectrometer was equipped with a 200 mW diode-pumped solid-state (DPSS) laser (Coherent Radiation Model 532) operating at 532 nm and a Brookhaven Instruments (BI 9000) correlator. The sample chamber included a thermostat, which controlled the temperature to within ± 0.02 °C. The intensity-intensity time

correlation functions were analyzed by the constrained regularization method developed by S.W. Provencher (see Provencher, S.W., "Makromol. Chem." 1979, 180, 201, *Comput. Phys. Commun.*, 1982, 27, 213, 229), known as the CONTIN method. From the SLS measurements, the weight-average molecular weight (M_w) of the macromolecules was determined, while DLS measurements provided information on particle size (in terms of hydrodynamic radius, R_h) and particle size distribution.

The resolution (R) for the test results was calculated using the following equation:

$$R = [2(t_2 - t_1)/(w_1 + w_2)] \quad (1)$$

where t_1 , t_2 , are the migration times and w_1 and w_2 are the temporal peak widths of the DNA fragments.

The DNA Sample

The separation of pBR322/Hae III is widely used for testing the sieving ability of separation media because pBR322/Hae III has 22 DNA fragments in the range of 8 bp to 587 bp. This interval covers most polymerase chain reaction (PCR)-amplified DNA chains produced for analysis of genetic diseases. In addition, the base pair intervals of adjacent fragments in this sample are small enough to test the sieving ability of separation media. Prior to the present invention, good separation of the 123 bp from 124 bp had not been achieved without using intercalating dyes in the buffer. In the presence of an intercalating dye in the running buffer, some polymers, such as HPMC, HEC, PEO, and PAM, have been successfully used to separate these two fragments. However, the presence of intercalating dyes in the

running buffer generally decreases the resolution of 434 bp and 458 bp.

For the first time, the present invention achieves total separation of 22 fragments in pBR322/Hae III. The problem encountered in previous attempts to separate the 22 fragments was the large difference in size between the small fragments and the large fragments. In order to detect the smallest 8 bp fragment, a relatively large amount of the large fragments has to be injected into the capillary, which may result in sample overloading and makes it difficult to achieve the necessary resolution if the detection is not sensitive enough. In the examples cited herein, a counting-mode photomultiplier tube ("PMT") was used for detection. The DNA sample used in Example 10 is described separately in Example 10.

Reagents

The reagents for the following examples were obtained from commercial sources. Acrylamide, ammonium persulfate, tris(hydroxymethyl)aminomethane (Tris), boric acid, ethylenediaminetetraacetic acid ("EDTA"), ethidium bromide and pBR322 Hae III digest were purchased from Sigma (St. Louis, MO). The pBR322/Msp I DNA was purchased from New England Biolabs (Beverly, MA). N, N, N',N'-tetramethylethylenediamine ("TEMED") was purchased from Fisher Scientific (Pittsburgh, PA). Poly (N-vinylpyrrolidone) ("PVP") with a molecular weight of 1,000,000 and polyacrylamide (PAM) with a molecular weight of 5,000,000-6,000,000 were purchased from Polysciences, Inc. (Warrington, PA). The electrophoresis buffer was 1 × TBE (89 mM Tris/89 mM boric acid/2.5 mM EDTA) with 1 µg/ml ethidium bromide. The two DNA samples were diluted to 10 µg/ml with water before use. The reagents used in Example 10 are described separately in Example 10.

Laser-Induced Fluorescence Detection System

A laser-induced fluorescence detection system was used in the examples for capillary electrophoresis detection. The system included a water-cooled Ar ion laser which was used to generate an excitation beam with wavelength (λ) of 488 nm and an incident power of about 5 mW. The laser beam was focused by a lens with a 25-cm focal length, reflected by a dichroic mirror (550DRLP, Omega Optical, Brattleboro, VT), and focused again using a 10× objective to a spot within the separation channel. The fluorescence was collected by the objective, passed through the dichroic mirror, and filtered by a bandpass filter (605DF50, Omega Optical, Brattleboro, VT) to the photomultiplier tube (“PMT”) (Hamamatsu R928, Hamamatsu Corporation, Bridgewater, NJ). Images of the capillary were made using a charge coupled device (CCD) camera (SONY SSC-M350, SONY Corporation, New York, NY). A white beam from the illuminator on the microscope illuminated the capillary. The image from the illuminated capillary was then focused by the objective, reflected by a slide in-and-out mirror, magnified by a Zoom 6000 System (D. O. Industries, Rochester, NY) and then detected by the CCD camera. The microscope and the CCD camera (Karl Zeiss, Melville, NY) provided good optical quality and fast alignment.

EXAMPLE 1

For this example, 1-ml solutions with desired concentrations of PVP and acrylamide were prepared in 1 × TBE and stored in a refrigerator overnight to ensure a homogeneous dissolution. The solutions were then purged for 30 minutes with high-purity nitrogen to

minimize dissolved oxygen. Polymerization was initiated by the addition of 1 μ l TEMED and 10 μ l 10 % w/v ammonium persulfate (“APS”) and allowed to proceed to completion for several hours. Laser light scattering studies showed that this procedure allowed the polymerization of PAM with an average molecular weight of about 400,000 g/mol without significant variations at different concentrations of acrylamide and PVP. For the purpose of comparison, pure PAM solutions were also synthesized under the identical conditions. The pure PAM solutions had about the same average molecular weight of 400,000 g/mol.

EXAMPLE 2

This example uses PAM and PVP to examine the combining of two different polymers to form an interpenetrating network. Due to the totally different chemical structures of PAM and PVP, interpenetrating networks cannot be prepared by dissolving PVP and PAM in 1 \times TBE buffer. Therefore, the compatibility of PAM and PVP was the first consideration in the preparation of a solution. Initially, PVP having a molecular weight of one million g/mol and PAM with a molecular weight of five million g/mol were dissolved in 1 \times TBE buffer. At PVP concentrations of 2%, 4% and 6% (all concentrations are w/v %), phase separation was observed when the PAM concentrations were 2%, 1% and 1%, respectively. Even for a solution mixture of PVP and PAM without phase separation, the DNA separation was still poor. The poor separation was attributed to micro-phase separation of the mixtures. To avoid this problem, PAM was synthesized in a PVP solution matrix so that the PAM chains were grown in a network of PVP chains. This produced more homogenous mixtures of PVP and PAM. Laser light scattering studies showed that interpenetrating networks of PVP and PAM were formed

by this synthesis. These interpenetrating networks were found to provide efficient DNA separation.

EXAMPLE 3

In this example, interpenetrating networks of PAM and PVP were used for separations of the 22 DNA fragments of pBR322/Hae III in the range of 8 to 587 bp. The separations were carried out in a 10/12 cm effective/total length capillary with a 100/365 μm ID/OD. The injection was performed at 50 V/cm for 3 seconds and the separation electric field strength was 100V/cm. The highest separation efficiency reached by the PAM/PVP networks had an order of 10^7 theoretical plate numbers per meter, as shown in Figures 1 and 2. By comparison, a separation medium containing 4% (i.e., w/v %) PAM and no PVP had little sieving ability of DNA fragments. PVP was then added to the 4% PAM in accordance with the method of the present invention in concentrations of 0.5%, 1%, 2% and 4%. The interpenetrating networks with PAM and PVP greatly increased the separation ability. These results are shown in Figures 1A to 1D. The difference in the migration times for the four samples is believed to be caused by the different mesh sizes of the four separation media. By varying the ratio of PVP to PAM, the mesh size of the separation media can be controlled.

EXAMPLE 4

This example demonstrates the effect of different concentrations of PVP in a PAM/PVP separation medium. For this example, the same DNA and separation conditions were used as in Example 3. A pure 2% (i.e., w/v %) PVP separation medium was tested and found to have

little sieving ability of DNA fragments. Interpenetrating networks were then prepared from 2% PVP and PAM concentrations of 1%, 2%, 4%, 6%. These networks were used as separation media and Figures 2A to 2D show that the sieving ability of 2% PVP had been greatly increased by the addition of PAM. The separations as shown in Figure 2A, with 2% PVP + 1% PAM were significantly better and faster than similar separations using PAM alone. For example, under the same separation conditions, resolution similar to those shown in Figure 2A could only be achieved by using at least a 6% PAM solution and with much longer run times. When pure PVP was used, the same separation as shown in Figure 2A could not be achieved even at a PVP concentration of 15 %. Further tests showed that a separation medium composition of 2% PVP and 1% PAM was the optimum combination for a fast separation of pBR322/HaeIII with good resolution of both 123/124 and 434/458 bp, as illustrated in Figure 3. At a PVP concentration of 4%, a PAM concentration of 0.5% also provided good separation. However, the separation run time was somewhat longer than the 2% PVP/1% PAM solution and about the same as the 2% PVP + 2% PAM solution mixture run time.

EXAMPLE 5

This example demonstrates the effect of the inner diameter of the capillary on DNA separation. Interpenetrating networks of 2% (i.e., w/v %) PVP + 1% PAM were used for separations of the 22 DNA fragments of pBR322/Hae III in the range of 8 to 587 bp. The separations were carried out in a 10/12 cm effective/total length capillary. The injections were performed at 50 V/cm for 3 seconds and the separation electric field strength was 100V/cm.

Figure 2A shows the test results for a 100 μm ID capillary. The resolutions of 123/124 bp and 434/458 bp were calculated to be 0.69 and 1.14 using equation (1). It was found that these resolutions could be increased by decreasing the inner diameter of the capillary. Figure 4B shows the results when the same 2% PVP + 1% PAM interpenetrating network was used as a separation medium and the inner diameter of the capillary was decreased from 100 μm to 50 μm . The resolutions of 123/124 bp and 434/458 bp were calculated and found to have increased to 0.87 and 1.54.

EXAMPLE 6

In this example, the effect of electric field strength was studied and it was found that the resolution could be improved further by varying the applied electric field. Interpenetrating networks of 2% (i.e., w/v %) PVP + 1% PAM were used for separations of the 22 DNA fragments of pBR322/Hae III in the range of 8 to 587 bp and the results are shown in Figures 4A to 4D. The separations were carried out in a 10/12 cm effective/total length capillary with a 50 μm ID. The injections were performed at 50 V/cm for 3 seconds. The separation electric field strengths were: 50V/cm for the tests reported in Figure 4A; 100V/cm for the tests reported in Figure 4B; 200V/cm for the tests reported in Figure 4C; and a gradient of from 200V/cm to 25V/cm with a duration time of 12 minutes and 25 V/cm thereafter for the tests reported in Figure 4D.

When the applied electric field strength was increased from 100 V/cm to 200 V/cm, the separation was completed within 6.5 min, as demonstrated in Figure 4C. While the resolution

of 123/124 bp was increased to 0.94, the resolution of 434/458 bp was decreased to 0.87. When the applied electric field strength was decreased from 100 V/cm to 50 V/cm, 30 minutes was required to complete the separation, as shown in Figure 4A. The resolution of 123/124 bp was decreased to 0.67 and the resolution of 434/458 bp was increased to 1.72. In order to achieve a better resolution of both 123/124 bp and 434/458 bp in a single run, a gradient electric field strength that changed with time was tried. For a linear electric field gradient from 200 V/cm to 25 V/cm with a time duration of 12 min being used, the separation was completed within 14 minutes with a resolution of 0.98 and 1.56 for 123/124 bp and 434/458 bp, respectively, as shown in Figure 4D.

Figure 4C shows that the separation medium of the present invention, achieved a separation time of about 6.5-minutes using a capillary with an effective separation length of 10 cm and an electric field strength of 200 V/cm. When the sampling frequency increased, the peak became lower and the smaller peaks became practically invisible. The faster separations were mainly due to the use of low concentrations of the separation medium. In addition, the entangled networks of the present invention have lower molecular weights than mediums that were previously used to achieve equivalent separation results. The lower concentration allows the DNA fragments to pass through the medium more quickly and, therefore, provide faster run times.

EXAMPLE 7

In this example, the sieving ability of a 2% (i.e., w/v %) PVP + 2% PAM matrix was

demonstrated by the CE separation of pBR322/Msp I DNA. The separation was carried out in a 10/12 cm effective/total length capillary with a 100/365 μm ID/OD. The injection was performed at 50 V/cm for 3 seconds and the separation electric field strength was 100V/cm. This sample contained four pairs of fragments with the same lengths: 26, 34, 147 and 160 bps. Even though the separation was completed in only 17 min, the two 160 bp fragments were still successfully separated with a resolution of 1.24. The results are shown in Figure 5.

EXAMPLE 8

In this example, the separation reproducibility for the separation of pBR322/Hae III was investigated and the test results are shown in Figure 6. For the test results shown in Figure 6A, the separation medium and the separation buffer were replaced after each run and the capillary was washed with 1 N HCl for 10 minutes between runs. The relative standard deviation of the migration time measured for each DNA fragment was less than 1% (i.e., w/v %) in seven runs, suggesting that the coating of the capillary inner wall was quite reproducible. For the test results shown in Figure 6B, the same capillary, buffer and separation medium of 2%PVP + 2% PAM were used for each of the test runs. For the first seven runs, the relative standard deviation of the migration time was still less than 1.4 %. After that, the migration time decreased. However, in the 14 consecutive runs, the separation of the pBR322/Hae III remained within acceptable limits.

EXAMPLE 9

For this example, PAM and copolymers of acrylamide (“AM”) and dimethylacrylamide

005090"0209050
("DMA") were prepared. Monomers of AM (or AM and DMA) with a total concentration of 1.4 M were mixed with TEMED, resulting in a TEMED concentration of 0.09 $\mu\text{l/ml}$ in the solution mixture. The solution mixture was then cooled in a 1 $^{\circ}\text{C}$ cold water bath and purged with ultra high purity helium for 5 hours to minimize dissolved oxygen. Polymerization was initiated by the addition of 50% ammonium persulfate ("APS") to a final APS concentration of 0.09 mg/ml and allowed to proceed to completion for 24 hours at 1 $^{\circ}\text{C}$. The solution was then diluted to 0.11 M (based on monomer concentration) and after shaking for three weeks, the solution was homogenous. Acetone was added to precipitate the polymer product in the solution. The polymer was then dried under vacuum. The copolymers of AM and DMA that were formed were characterized. An AM to DMA ratio of 2.94, 1.85 and 1.06 was determined by NMR. The molecular weight of the copolymers was estimated to be similar to that of PAM (with M_v of about 2.2×10^6 Da (or 2.2 mDa)).

EXAMPLE 10

In this example, copolymers of AM and DMA with molar ratios of 3:1, 2:1, 1:1, respectively, as well as PAM were used to separate single-stranded DNA fragments.

The electrophoresis buffer for the separation of single-stranded DNA was 1 \times TTE (50 mM Tris/50 mM TAPS/2.0 mM EDTA) for the anode and 1 \times TTE/5M urea for the cathode. All separation media were prepared in 1 \times TTE/5M urea buffer. To prepare a separation medium, 1 \times TTE/5M urea was added to a known weight of dry PAM or the random copolymers P(AM/DMA) to the desired concentration. After the PAM or the random copolymer was

swollen by the buffer overnight, the solution was vortexed for 30 seconds, twice a day with at least a 6 hour interval. After 2-3 days, the solution was degassed by ultracentrifugation (7×10^4 g) before use.

Sequencing reactions were performed by using an ABI PRISM™ Dye Primer (-21 M13 forward) Cycle Sequencing Ready Reaction Kit with AmpliTaq® DNA Polymerase, FS (PE Biosystems/Perkin-Elmer Corp., Foster City, CA) on a pGEM3Zf(+) double-stranded template. Single dye-labeled sequencing reactions were performed using the FAM labeled primer and the C termination mix. The temperature cycling protocol was carried out using the GeneAmp PCR System 2400 (PE Biosystems/Perkin-Elmer Corp., Foster City, CA) with 15 cycles of 10s at 95 °C, 5 sec. at 50 °C and 1 min. at 70 °C, followed by 15 cycles of 10 sec. at 95 °C and 1 min at 70 °C. The reaction products were purified by ethanol precipitation and resuspended in 20 µl deionized formamide.

The separation of single-stranded DNA fragments with p(AM/DMA) 2:1 was compared with PAM under the same separation conditions: 50:40 cm total/effictive capillary length, 75µm capillary inner diameter, 300 V/cm for 15 sec. injection and 150 V/cm for separation. When PAM was used, the inner wall of the capillary was covalently coated with PAM using the protocol described by Hjerten (*J. Chromatogr.*, 1985, 347, 191-198). When the copolymer was used, the capillary was simply washed with 1 M HCL for 10 minutes and the copolymer could coat the capillary dynamically, thereby avoiding the tedious covalent coating procedure. The

results are shown in Figures 7A-D. It was found that (PAM/DMA) 2:1 provided better resolution for the single-stranded DNA fragments than PAM.

For the purpose of a fast separation with long read length, the separation conditions were further optimized to 2.5 w/v % copolymer concentration, 50/40 cm total/effective capillary length, 75 μ m capillary inner diameter, 150 V/cm for 5 sec. injection, and 150 V/cm for separation. Under the optimized conditions, the use of P(AM/DMA) 3:1 has achieved the best results for the separation of single-stranded DNA fragments, which is shown in Figure 8. A one base resolution of 0.55 and up to 699 bases and two base resolution of 0.61 up to 963 bases have been achieved without the aid of algorithms. It should be noted that the PAM and P(AM/DMA) had similar molecular weights. The performance of P(AM/DMA) could be further improved by using molecular weight copolymers and other random copolymers (slightly incompatible, one or both surface active) should have similar improvements when compared with the corresponding homopolymers.

Thus, while there have been described the preferred embodiments of the present invention, those skilled in the art will realize that other embodiments can be made without departing from the spirit of the invention, and it is intended to include all such further modifications and changes as come within the true scope of the claims set forth herein.

We claim:

1. A polymer solution for the efficient separation of charged macromolecules by electrophoresis comprising a plurality of polymers, wherein said polymers are different, do not phase separate when dissolved in solution and are entangled to form an interpenetrating network.

2. A polymer solution in accordance with Claim 1, wherein said polymers are neutral and water-soluble.

3. A polymer solution in accordance with Claim 1, wherein at least one of said polymers is polyacrylamide ("PAM"), N-substituted PAM, N,N-disubstituted PAM, modified polysaccharides, polyethylene oxide ("PEO"), polyvinylpyrrolidone ("PVP"), polyvinylalcohol PVA, polyethylene glycol ("PEG"), or a random, a graft or a block copolymer based on the backbone monomer segments thereof, wherein nitrogen substitutes are selected from the group consisting of C₁ to C₃ alkyl, hydroxyl-substituted C₁ to C₃ alkyl, and methoxy-substituted C₁ to C₃ alkyl.

4. A polymer solution in accordance with Claim 3, wherein said random, graft or block copolymer is EPE-type Pluronics, N,N-diethylacrylamide ("P(DMA/DEA)"), a copolymer of poly(N-isopropylacrylamide) densely grafted with short poly(ethylene oxide) ("PNIPAM-g-PEO") or polyacrylamide-co-allyl-β-D-glucopyranoside ("P(AM/AG)").

5. A polymer solution in accordance with Claim 3, wherein said polysaccharides are selected from the group consisting of liquified agrose, methylcellulose ("MC"), hydroxyethylcellulose ("HEC"), hydroxypropyl-methyl-cellulose ("HPMC"), hydroxypropylcellulose ("HPC"), glucomannan, galactonmannan and dextran.

5 6. A polymer solution in accordance with Claim 1, wherein at least one of said polymers is a silica-absorbing polymer that suppresses electrophoendoosmotic flow and charged macromolecule-silica interactions.

7. A polymer solution in accordance with Claim 6, wherein said silica-absorbing polymer is selected from the group consisting of PVP, PEO, EPE-type Pluronics, N-substituted
10 PAM and N,N-disubstituted PAM, and wherein nitrogen substitutes are selected from the group consisting of C₁ to C₃ alkyl, hydroxyl-substituted C₁ to C₃ alkyl, and methoxy-substituted C₁ to C₃ alkyl.

8. A polymer solution in accordance with Claim 1, wherein said interpenetrating network has a more expanded structural formation than the entanglement structure of a
15 corresponding homopolymer solution, and has a larger effective size than that of a corresponding homopolymer solution, representing an effective entanglement network greater than that of the corresponding homopolymers, and wherein said interpenetrating network has a lower molecular weight per volume than the corresponding homopolymers.

9. A polymer solution in accordance with Claim 1, wherein said interpenetrating network is prepared by synthesizing a first polymer in a matrix of a second polymer solution.

10. A polymer solution in accordance with Claim 1, wherein said polymer solution provides at least a 500-base read length in one run for a single-stranded DNA separation.

5 11. A polymer solution for the efficient separation of charged molecules by electrophoresis comprising a plurality of stretched polymer chains having polymer chain entanglement times greater than the corresponding linear homopolymer solution, wherein said polymer chains comprise the same polymer or a plurality of different polymers.

10 12. A polymer solution in accordance with Claim 11 wherein said polymer chains entangle to form an interpenetrating network in solution.

13. A polymer solution in accordance with Claim 11, wherein said polymers are PAM and PVP or PDAM and PVP.

14. A polymer solution in accordance with Claim 11, wherein said polymer chains comprise a random copolymer.

15 15. A polymer solution in accordance with Claim 14, wherein said random copolymer comprises a monomer taken from PAM, N-substituted PAM, N,N-disubstituted PAM, modified polysaccharides, PEO, PVP, PVA or PEG.

16. A polymer solution in accordance with Claim 15, wherein said random copolymer further comprises a silica-absorbing segment taken from PVP, PEO, EPE-type Pluronic, N-substituted PAM or N,N-disubstituted PAM, and wherein nitrogen substitutes are selected from the group consisting of C₁ to C₃ alkyl, hydroxyl-substituted C₁ to C₃ alkyl, and methoxy-substituted C₁ to C₃ alkyl.

17. A polymer solution in accordance with Claim 14, wherein said random copolymer comprises acrylamide ("AM") and dimethyl acrylamide ("DMA").

18. A polymer solution in accordance with Claim 11, wherein said polymer chains comprise a graft copolymer.

19. A polymer solution in accordance with Claim 18, wherein said graft copolymer comprises a monomer taken from PAM, N-substituted PAM, N,N-disubstituted PAM, modified polysaccharides, PEO, PVP, PVA or PEG.

20. A polymer solution in accordance with Claim 19, wherein said graft copolymer further comprises a silica-absorbing segment taken from PVP, PEO, EPE-type Pluronic, N-substituted PAM or N,N-disubstituted PAM, and wherein nitrogen substitutes are selected from the group consisting of C₁ to C₃ alkyl, hydroxyl-substituted C₁ to C₃ alkyl, and methoxy-substituted C₁ to C₃ alkyl.

21. A polymer solution in accordance with Claim 18, wherein said graft copolymer comprises PNIPAM-g-PEO.

22. A polymer solution in accordance with Claim 14, wherein said polymer chains comprise a very weakly cross-linked microgel.

5 23. A polymer solution in accordance with Claim 22, wherein said very weakly cross-linked microgel comprises a monomer taken from PAM, N-substituted PAM, N,N-disubstituted PAM, modified polysaccharides, PEO, PVP, PVA or PEG.

10 24. A polymer solution in accordance with Claim 23, wherein said very weakly cross-linked microgel further comprises a silica-absorbing segment taken from PVP, PEO, EPE-type Pluronics, N-substituted PAM or N,N-disubstituted PAM, and wherein nitrogen substitutes are selected from the group consisting of C₁ to C₃ alkyl, hydroxyl-substituted C₁ to C₃ alkyl, and methoxy-substituted C₁ to C₃ alkyl.

25. A polymer solution in accordance with Claim 22, wherein said very weakly cross-linked microgel comprises PAM and a hydrophilic cross-linker.

15 26. A polymer solution in accordance with Claim 25, wherein said hydrophilic cross-linker is PEO diacrylate.

27. A polymer solution in accordance with Claim 11, wherein said polymer solution provides at least a 500-base read length in one run for a single-stranded DNA separation.

ABSTRACT

The present invention is a polymer solution for the efficient separation of charged macromolecules by electrophoresis that includes a plurality of polymers. These polymers are entangled to form an interpenetrating network with greater entanglement times than corresponding homopolymers of the same length. These polymers are different and do not phase separate when dissolved in solution. Preferred polymers are PAM and PVP or PDAM and PVP. The polymer solutions of the present invention provide at least a 500-base read length in one run for a single-stranded DNA separation.

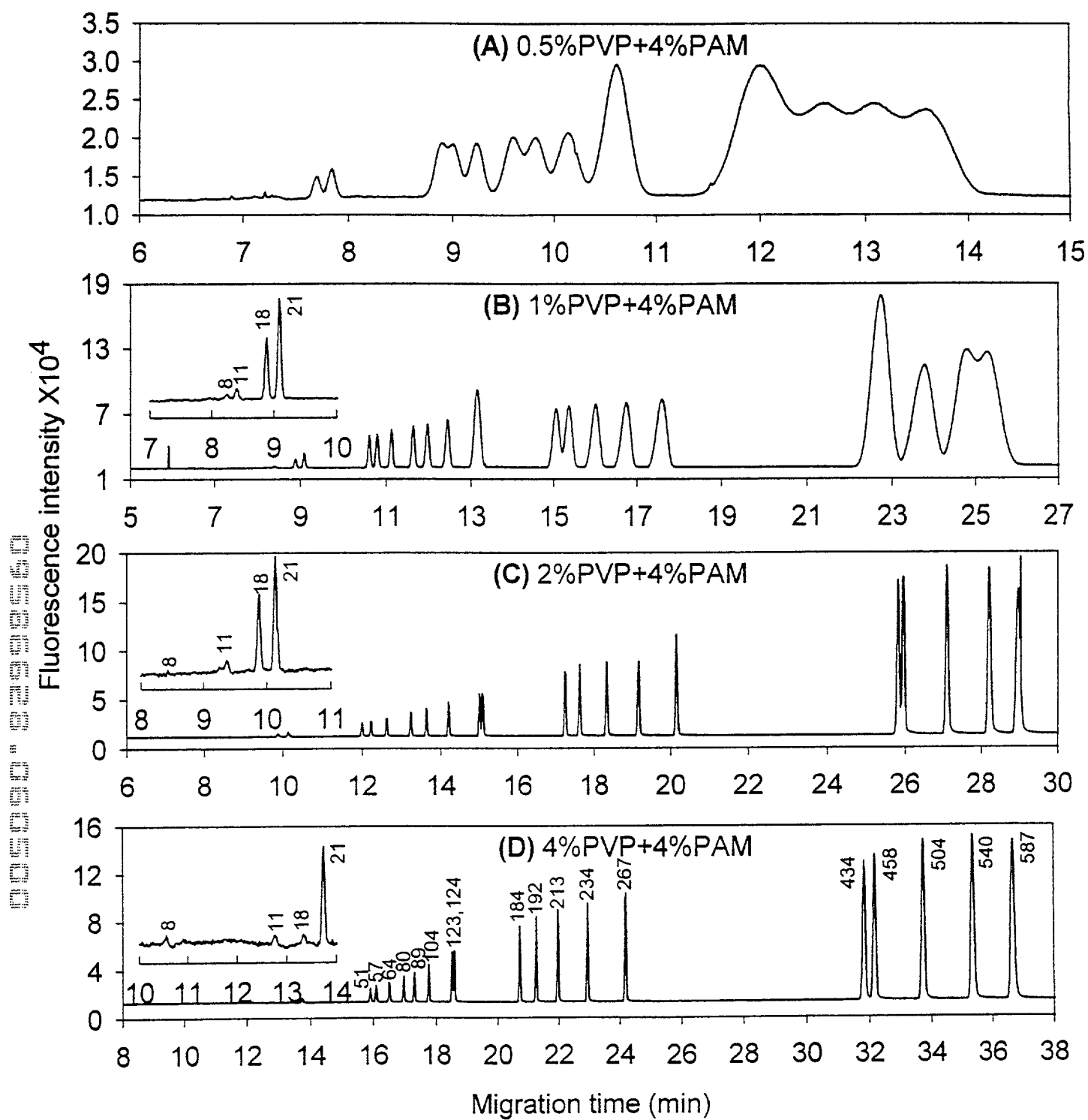


FIG. 1

A 3D plot showing the relationship between Migration time (min) on the vertical axis, PVP concentration (w/v, %) on the horizontal axis, and PAM concentration (w/v, %) on the depth axis. The vertical axis ranges from 10 to 60 minutes. The horizontal axis (PVP concentration) ranges from 6 to 0. The depth axis (PAM concentration) ranges from 0 to 10. Two lines are plotted, representing different experimental conditions. The lines intersect, showing that migration time is influenced by both PVP and PAM concentrations. Dotted lines are used to project the data points onto the base plane.

PVP concentration (w/v, %)	PAM concentration (w/v, %)	Migration time (min)
5.5	0.5	26
5.0	0.5	29
4.5	0.5	39
3.5	0.5	16
3.0	0.5	20
2.5	0.5	26
2.0	0.5	42
1.5	0.5	17
1.0	0.5	11
0.5	0.5	28
0.0	0.5	32
0.5	1.5	41
0.0	1.5	48
0.5	2.5	34
0.0	2.5	44
0.5	3.5	40
0.0	3.5	60
0.5	4.5	50
0.0	4.5	60
0.5	5.5	49
0.0	5.5	56

FIG. 3

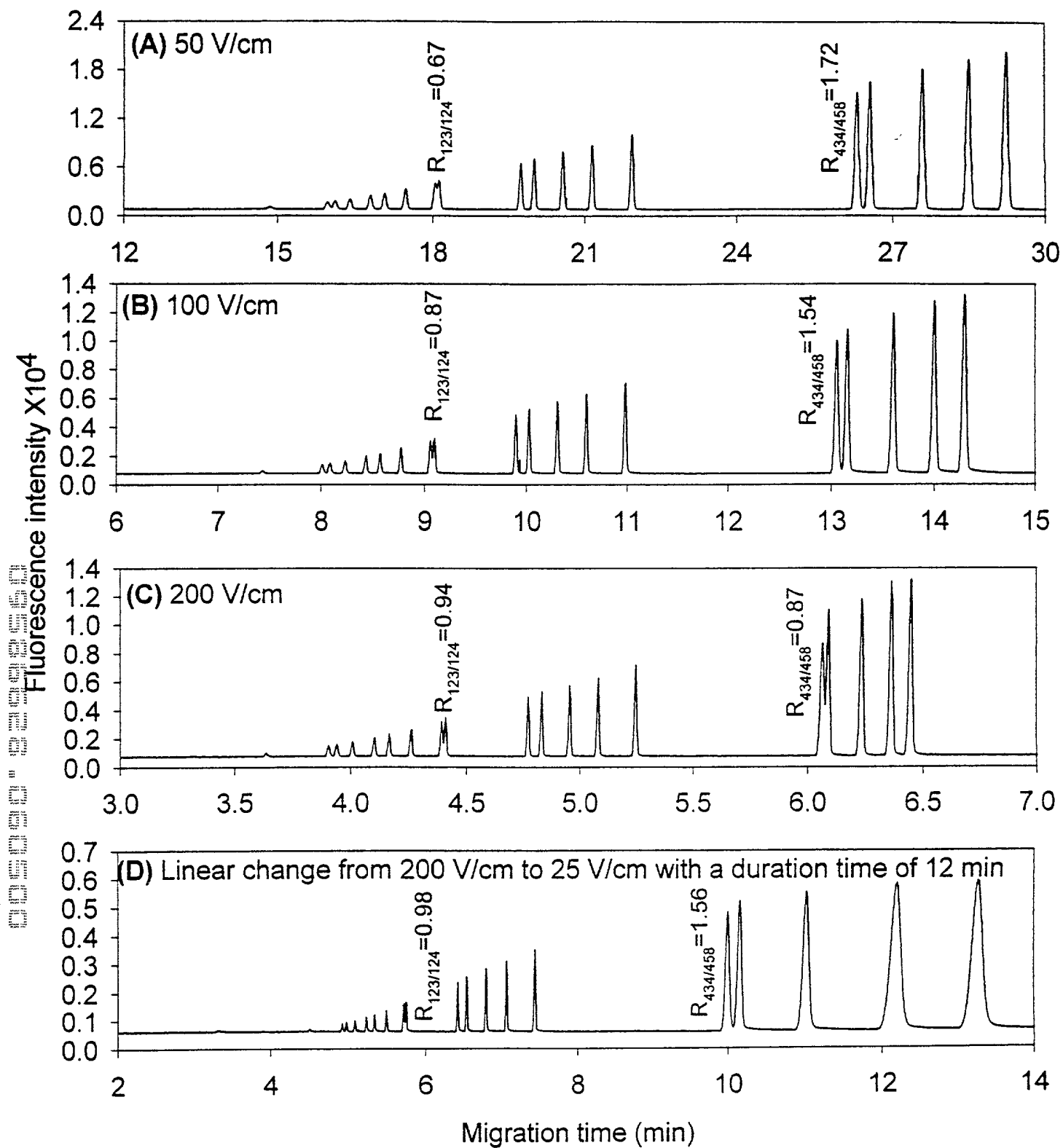


FIG. 4

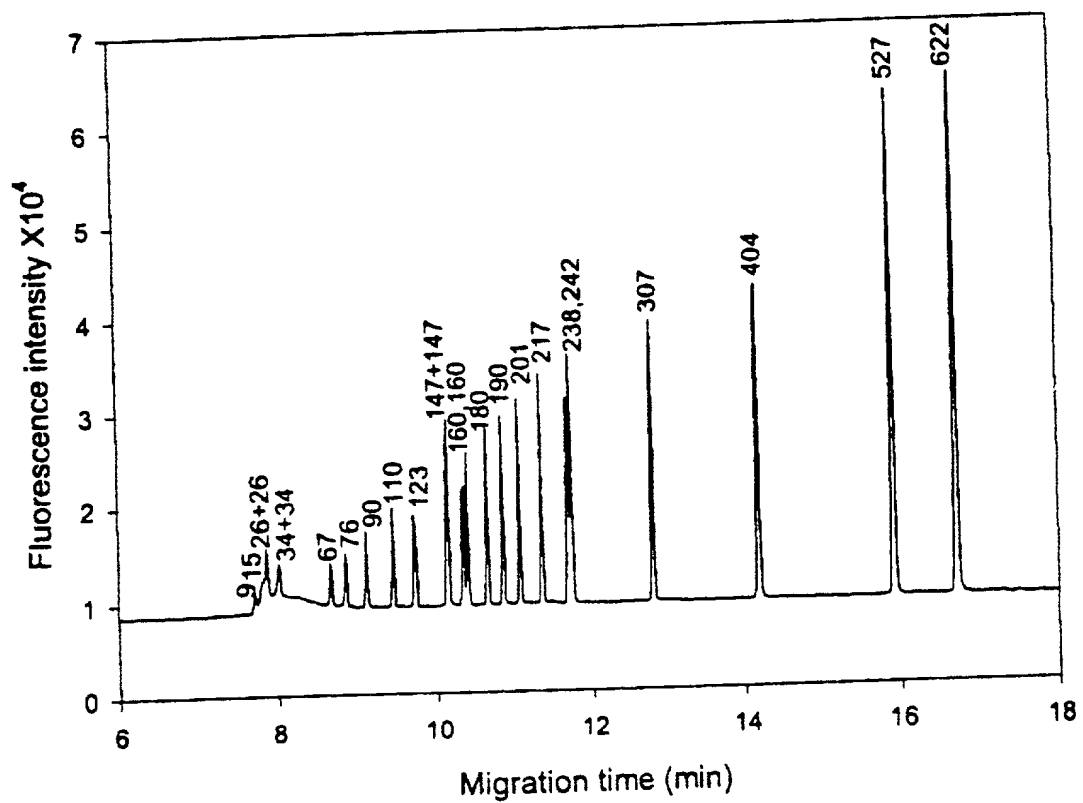


Fig. 5

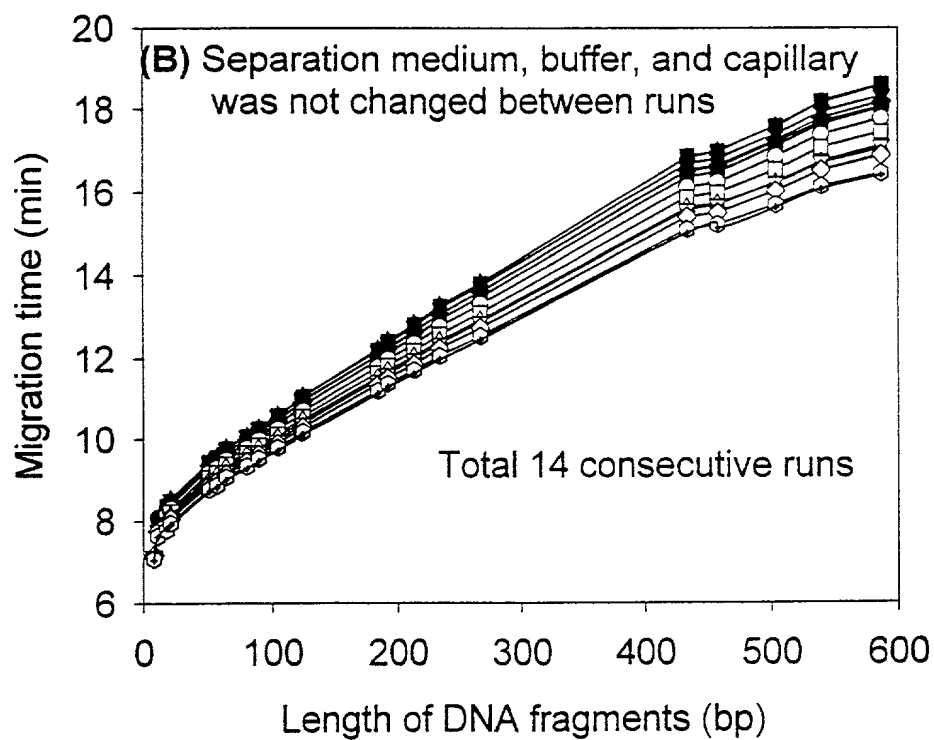
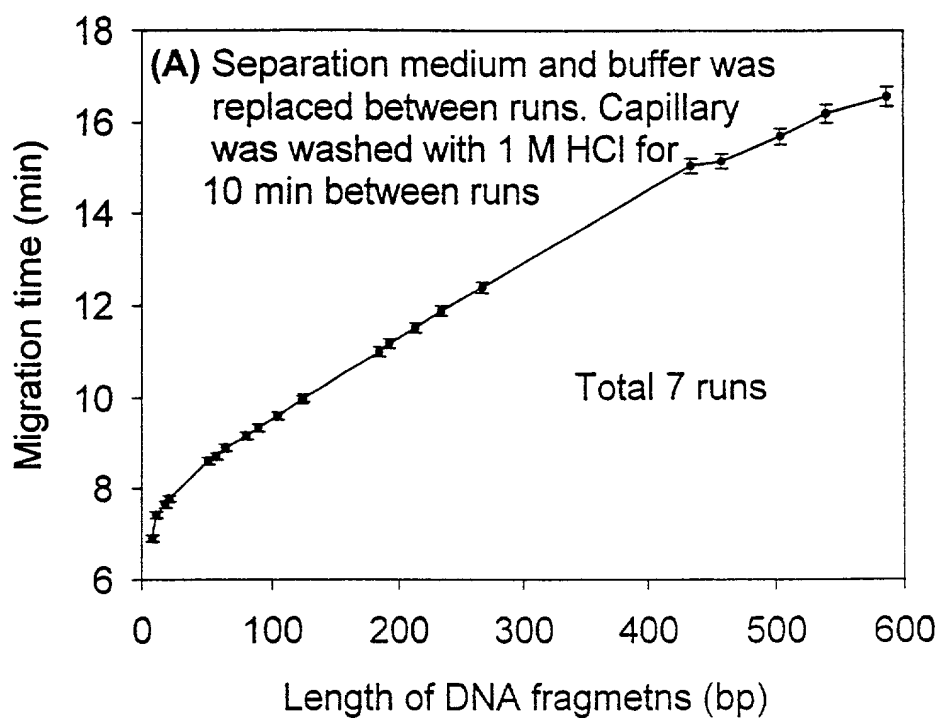


FIG. 6

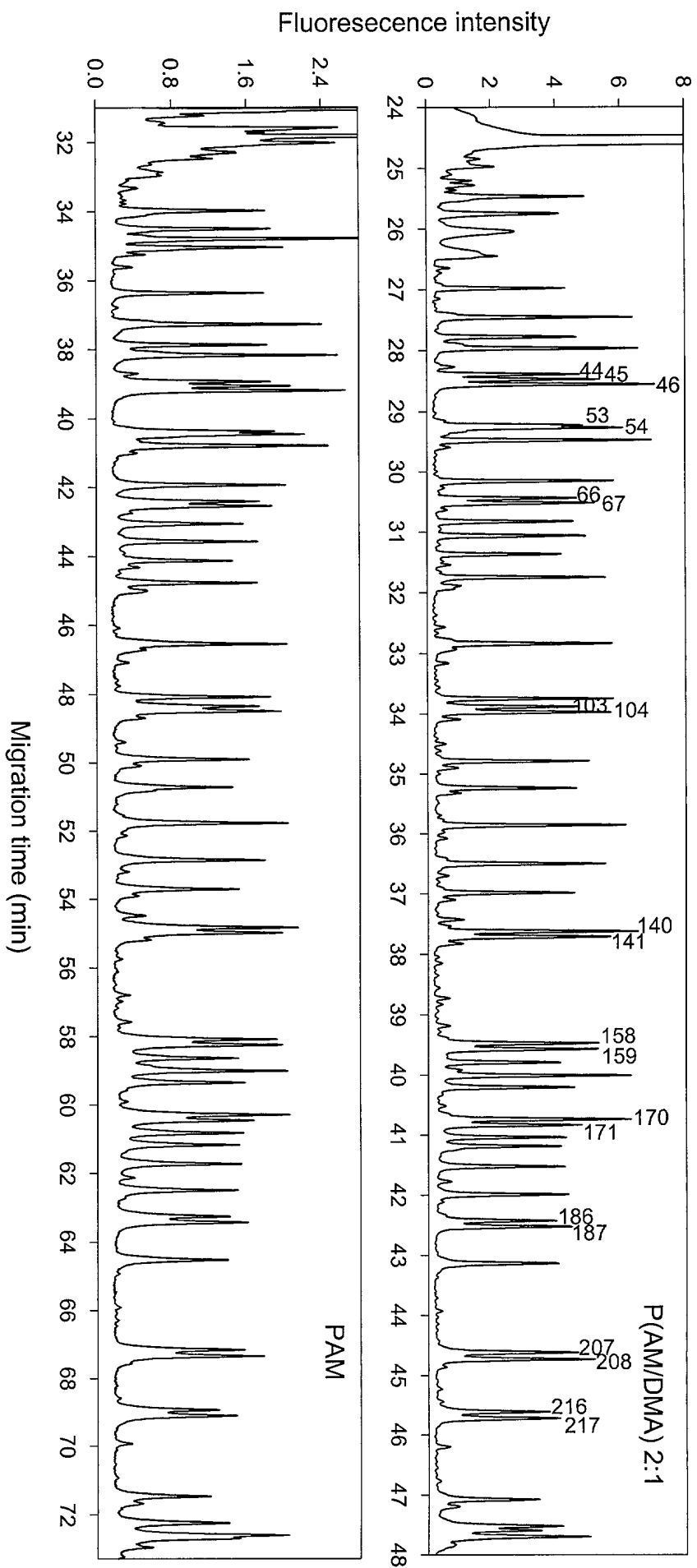


Fig 7A

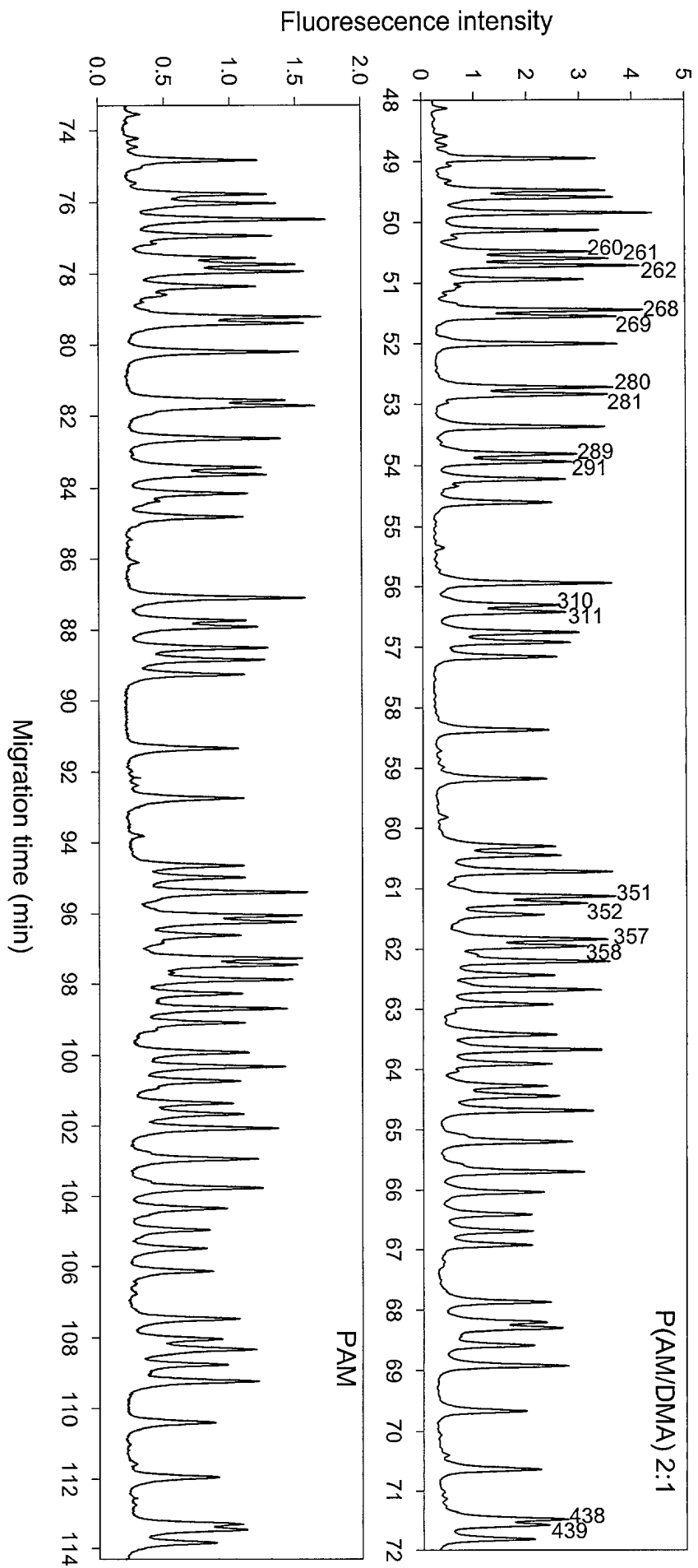


Fig 7B

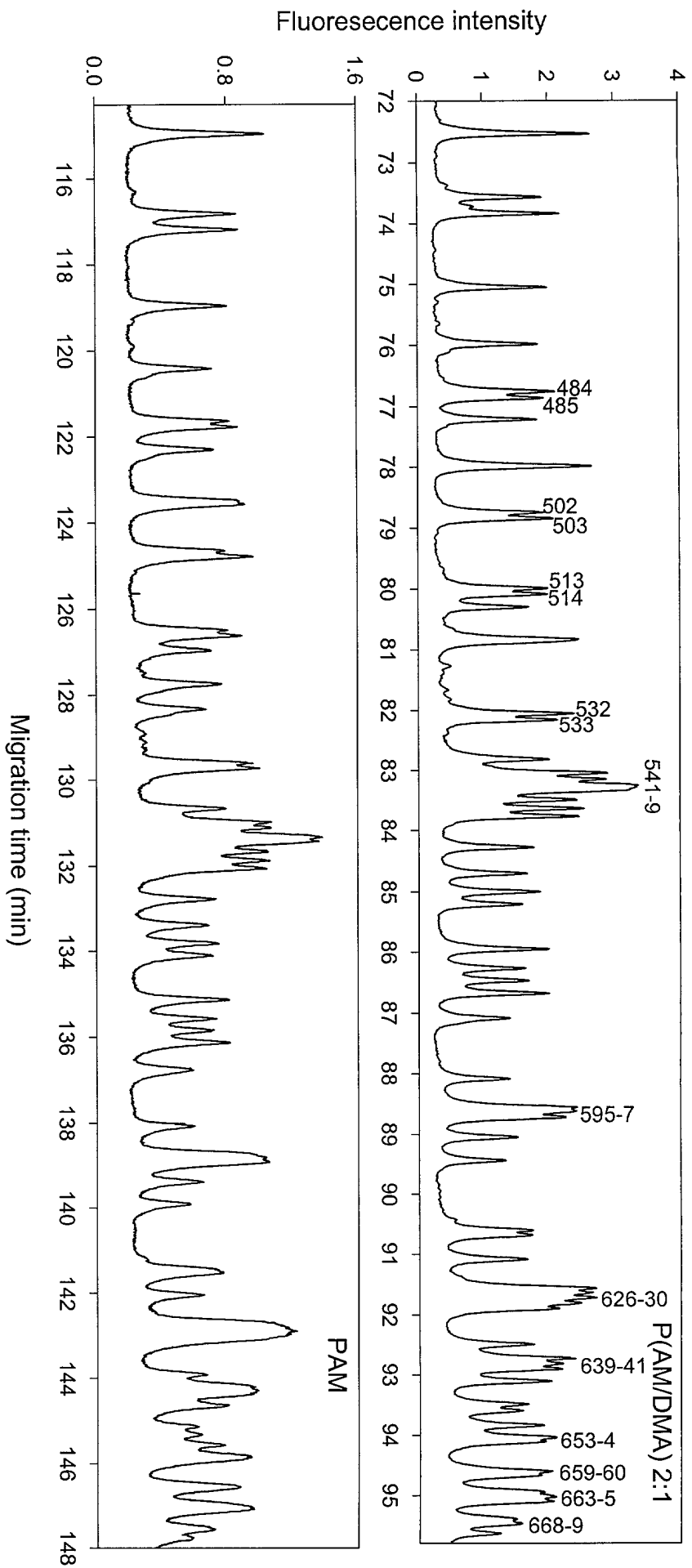


Fig 7C

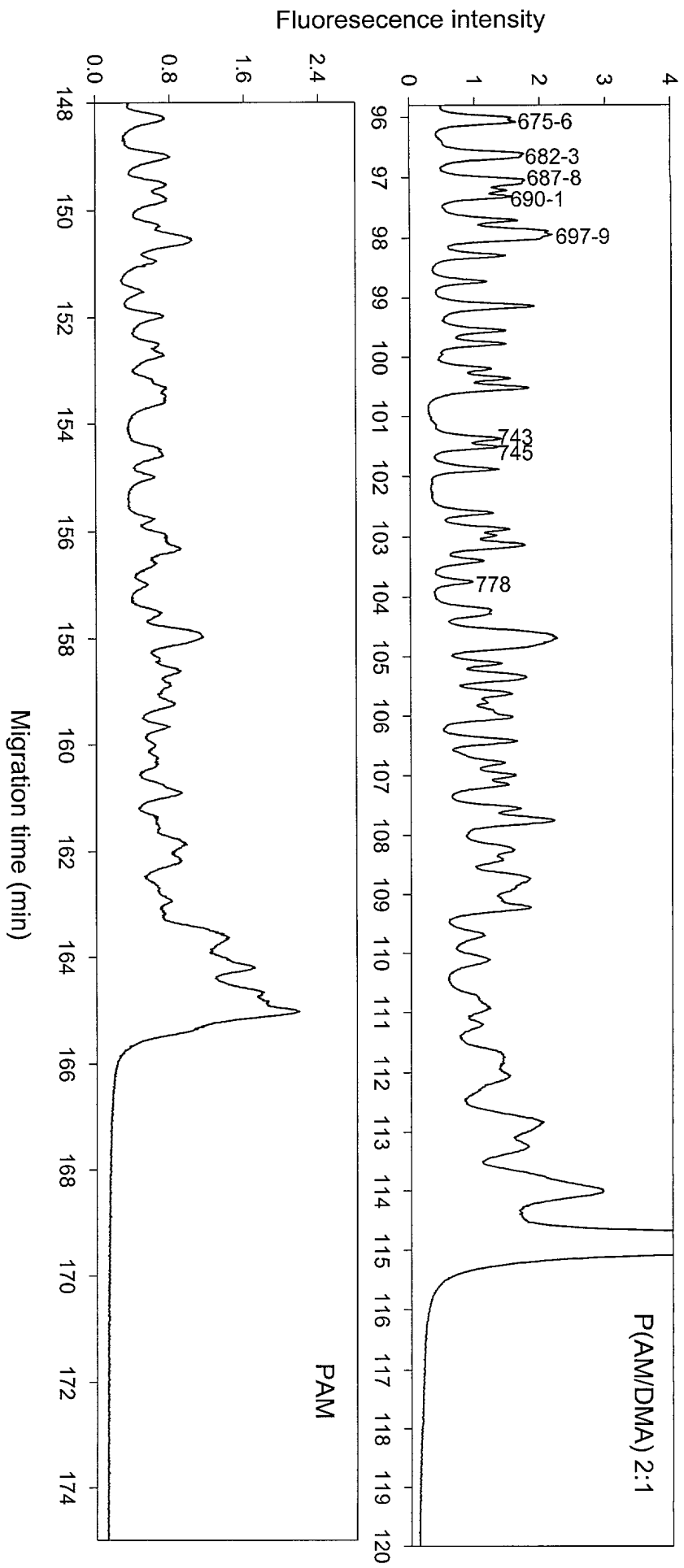


Fig 7D

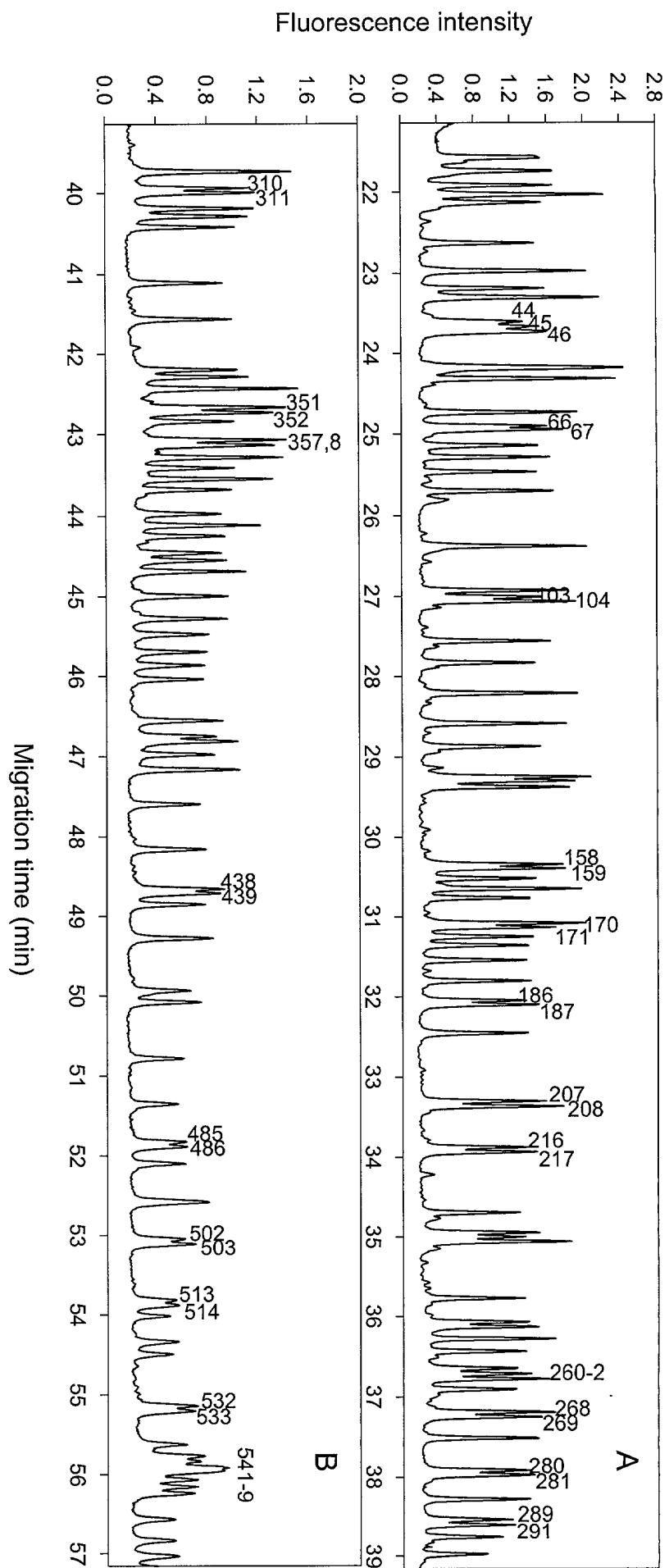


Fig 8A

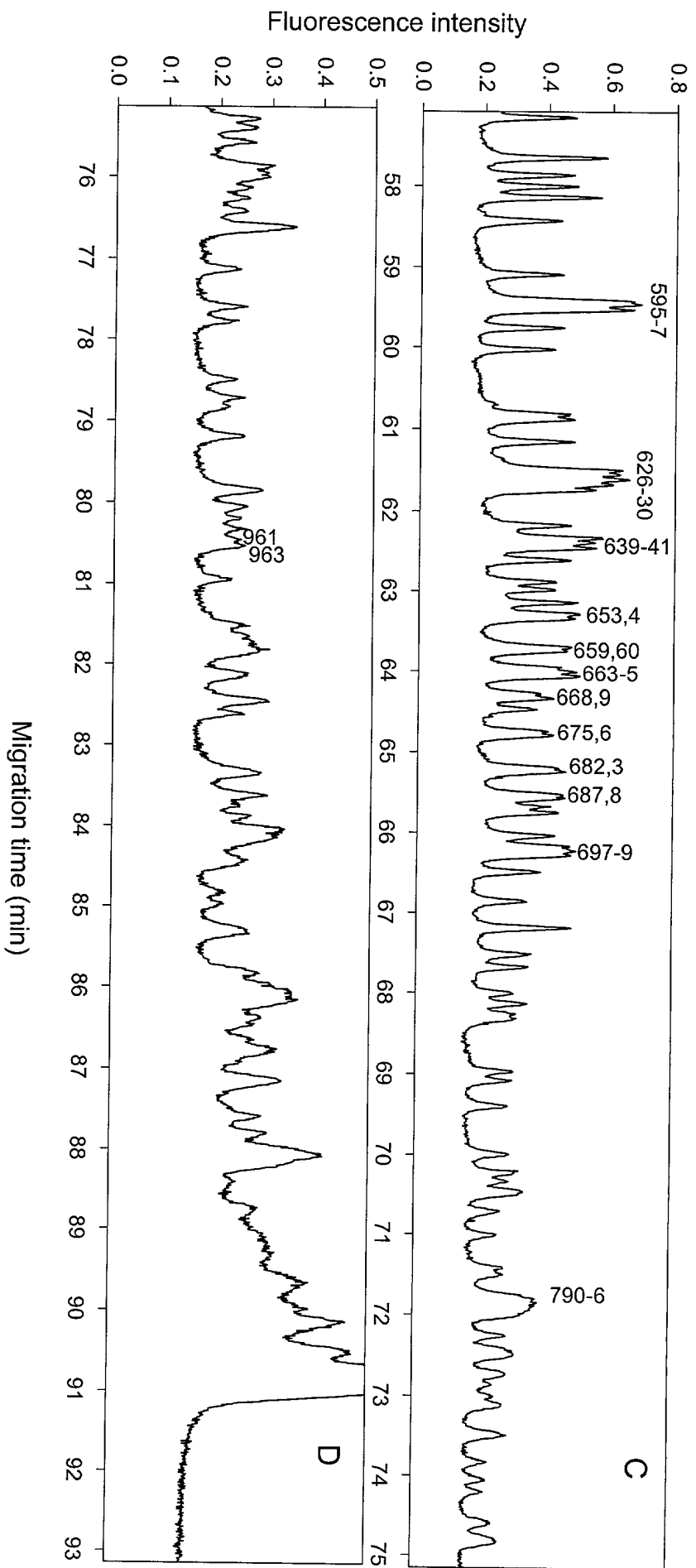


Fig 8 B

COMBINED DECLARATION AND POWER OF ATTORNEY

(ORIGINAL, DESIGN, NATIONAL STAGE OF PCT, SUPPLEMENTAL,
DIVISIONAL, CONTINUATION OR CIP)

As a below named inventor, I hereby declare that:

TYPE OF DECLARATION

This declaration is of the following type: *(check one)*

- | | |
|--|---|
| <input checked="" type="checkbox"/> Original | <input type="checkbox"/> National Stage PCT |
| <input type="checkbox"/> Supplemental | <input type="checkbox"/> Divisional |
| <input type="checkbox"/> Design | <input type="checkbox"/> Continuation |
| | <input type="checkbox"/> Continuation-in-Part (CIP) |

INVENTORSHIP IDENTIFICATION

NOTE: *If the inventors are each not the inventors of all the claims an explanation of the facts, including the ownership of all the claims at the time the last claimed invention was made, should be submitted.*

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

**POLYMER SOLUTION FOR SEPARATION OF CHARGED
MACROMOLECULES BY ELECTROPHORESIS**

the specification of which: *(complete (a), (b) or (c))*

- (a) ☒ is attached hereto.
- (b) ☐ was filed on _____ as
☐ Serial No. **08/**_____ or
☐ Express Mail No. _____, as Serial No. not yet known
and was amended on _____. *(If applicable)*
- (c) ☐ was described and claimed in PCT International Application No. **PCT/** _____
filed on _____ and as amended under PCT Article 19 on _____. *(If any)*

ACKNOWLEDGMENT OF REVIEW OF PAPERS AND DUTY OF CANDOR

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above, and that the filing of said specification, if heretofore filed, was authorized by me.

I acknowledge the duty to disclose information which is material to the patentability of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a).

CLAIM OF PRIORITY OF EARLIER FOREIGN APPLICATION(S) UNDER 35 U.S.C. §119(a)-(d)

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed:

(List prior foreign/PCT application(s) filed within 12 months (6 months for design) prior to this U.S. application.)

NOTE: Where item (c) is entered above and the International Application which designated the U.S. claimed priority check item (e), enter the details below and make the priority claim.

COUNTRY (orPCT)	APPLICATION NO.	DATE OF FILING (Day/Month/Year)	PRIORITY CLAIMED UNDER 35 USC §119	
			<input type="checkbox"/> YES	<input type="checkbox"/> NO
			<input type="checkbox"/> YES	<input type="checkbox"/> NO

CLAIM FOR BENEFIT OF PRIOR U.S. PROVISIONAL APPLICATION(S) UNDER 35 U.S.C. §119(e)

I hereby claim the benefit under Title 35, United States Code, §119(e) of any United States provisional application(s) listed below:

(List prior U.S. provisional applications.)

PROVISIONAL APPLICATION NO.	FILING DATE (Day/Month/Year)

CLAIM FOR BENEFIT OF EARLIER U.S./PCT APPLICATION(S) UNDER 35 U.S.C. 120

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) or PCT international application(s) designating the United States of America that is/are listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in such prior application(s) in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application(s) and the national or PCT international filing date of this application:

(List prior U.S. applications or PCT international applications designating the U.S. for benefit under 35 U.S.C. §120.)

U.S. APPLICATIONS

STATUS (Check One)

U.S. SERIAL NO.	U.S. FILING DATE (Day/Month/Year)	Patented	Pending	Abandoned
0 /		<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
0 /		<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

PCT APPLICATIONS DESIGNATING THE U.S.

STATUS (Check One)

PCT APPLN. NO.	PCT FILING DATE (Day/Month/Year)	U.S. SERIAL NOS ASSIGNED (If any)	Patented	Pending	Abandoned
PCT/			<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
PCT/			<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

35 USC 119 PRIORITY CLAIM, IF ANY, FOR ABOVE LISTED U.S./PCT APPLICATIONS

PRIORITY APPLICATION NO.	PRIORITY COUNTRY	FILING DATE (Day/Month/Year)	ISSUE DATE (Day/Month/Year)

POWER OF ATTORNEY

As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office in connection therewith:

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DECLARATION

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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